



Isolation and structures of grammistins, peptide toxins from the skin secretion of the soapfish *Grammistes sexlineatus*

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Received 4 February 1999; accepted 12 April 1999

Abstract

Two peptide toxins (named grammistins Gs 1 and Gs 2) with hemolytic and ichthyotoxic activities were isolated from the skin secretion of the soapfish *Grammistes sexlineatus*. Grammistin Gs 2 showed 6–11× higher hemolytic activity and 10× higher ichthyotoxicity than grammistin Gs 1. The complete amino acid sequences of Gs 1 comprising 25 residues and Gs 2 comprising 24 residues were determined. Although a search by the database failed to find any homologous toxins from other sources, the grammistins were similar in secondary structures as well as biological activities to the two classes of peptide toxins, melittin from the bee venom and pardaxins from the skin secretion of two species of soles. CD experiments and helical wheel projections showed that the grammistins were randomly coiled in distilled water but formed amphiphilic α -helices in the presence of SDS micelles. In addition, they were found to be surface seeking peptides by the Eisenberg plot and assumed to exist as aggregates of 3–4 molecules. Interestingly, grammistin Gs 2 is much more abundant in amphiphilic α -helices and much higher in biological activities than melittin and pardaxins as well as grammistin Gs 1. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Several groups of fish are known to release a surfactant secretion from the skin which makes the surrounding seawater foamy (Hashimoto, 1979; Tachibana, 1988). They commonly possess toxins with both ichthyotoxic and hemolytic activities in their skin secretion, presumably as defense substances against predators. The following toxins have so far been chemically well-characterized: pahutoxins (choline esters of fatty acids; Boylan and Scheuer, 1967; Goldberg et al., 1982; Fusetani and Hashimoto, 1987) from boxfishes; pavoninins (steroid glycosides; Tachibana et al., 1984, 1985), mosesins (steroid glycosides; Tachibana and Gruber, 1988) and pardaxins (peptides; Thompson et al., 1986, 1988; Shai et al., 1988) from two species of soles belonging to the genus *Pardachirus*; lipogrammistin-A (macrocyclic polyamine; Onuki et al., 1993) from the soapfish *Diploprion bifasciatum*. Despite the isolation and elucidation of lipogrammistin-A, the major toxins in soapfishes, members of the family Grammistidae, are surfactant peptides, called grammistins after the family name. Grammistins are universally distributed in all members (9 species) of Grammistidae (Hashimoto, 1979; Tachibana, 1988). Even in the case of *D. bifasciatum* containing lipogrammistin-A, about the half of the biological activity is ascribable to grammistins (Oshima et al., 1974). It should be noted that the four grammistin components purified from *Pogonoperca punctata* (Hashimoto and Oshima, 1972; Oshima and Hashimoto, 1976) and the three components from *Grammistes sexlineatus* (Oshima et al., 1974) have all been suggested to be not simple peptides but peculiar peptides with tertiary amine or quaternary ammonium base moieties. Besides soapfishes, coral-gobies of the genus *Gobiodon* (Hashimoto et al., 1974; Shiomi and Hashimoto, 1976) and the clingfish *Diademichthys lineatus* (Hori et al., 1979) have also been reported to contain grammistin-like toxins in their skin secretion.

Although grammistins and grammistin-like toxins appear to be chemically fascinating substances, their structures have remained unknown for a long time. Thus, this study was initiated to elucidate the structures of grammistins, using *G. sexlineatus* as a sample fish. Interestingly, the two grammistin components (named grammistins Gs 1 and Gs 2) isolated were established to be simple peptides, differing from the previous suggestions (Oshima et al., 1974). Furthermore, they were found to be surface seeking peptides with amphiphilic α -helices, similar to pardaxins from the sole skin secretion (Thompson et al., 1986, 1988; Shai et al., 1988) and melittin from the bee *Apis mellifera* venom (Habermann, 1972). We report here the isolation and the primary and secondary structures of grammistins Gs 1 and Gs 2 from the skin secretion of *G. sexlineatus*.

2. Materials and methods

2.1. Fish

Specimens of *G. sexlineatus* were purchased from Izu Chuo Suisan, Izu-

Nagaoka, Shizuoka Prefecture, Japan. Live specimens were frozen at Izu Chuo Suisan, shipped to our laboratory and stored at -20°C until used.

2.2. Isolation procedure

The skin secretion (10.6 g) was scraped off from the body surface of five fish (12–19 g in body weight and 9–12 cm in total length). It was suspended in 100 ml of 0.1% aqueous acetic acid and extracted three times with an equal volume of 1-butanol saturated with 0.1% aqueous acetic acid. The combined 1-butanol layer was evaporated to dryness, suspended in 50 ml of 0.1% aqueous acetic acid and defatted three times with an equal volume of diethyl ether. The aqueous layer (crude toxin) thus obtained was evaporated to dryness and dissolved in 30 ml of a mixture of methanol and 1% aqueous acetic acid (3:1, v:v). This solution (about 15 ml at a time) was subjected to gel filtration on a Sephadex LH-20 column (2.5×90 cm; Pharmacia, Uppsala), which was eluted with the same methanol–acetic acid solvent. Fractions of 8 ml were collected at a flow rate of about 35 ml/h and measured for absorbance at 258 nm and hemolytic activity against horse erythrocytes. Grammistin-containing fractions were pooled and evaporated to dryness. The dried material was dissolved in 12 ml of 0.1% trifluoroacetic acid (TFA) and a 0.1 ml portion was applied at a time to reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan). Elution was achieved at a flow rate of 1 ml/min by a gradient of acetonitrile (see Fig. 2 for details) in 0.1% TFA. Peptides were continuously monitored at 258 nm with an UV detector. Finally, two active fractions (Frs. I and II, see Fig. 2) were separately subjected to reverse-phase HPLC on a TSKgel ODS-80Ts column (0.46×15 cm; Tosoh) to remove impurities. The column was eluted under the same conditions as adopted for the above reverse-phase HPLC on TSKgel ODS-120T. Peptides were monitored at 220 nm with a UV detector.

2.3. Assay of hemolytic and ichthyotoxic activities

Hemolytic activity against horse, cow, sheep or rabbit erythrocytes was determined as described elsewhere (Shiomi et al., 1985). In brief, a mixture of sample solution (3 ml) and 2% erythrocyte suspension (1 ml), made in 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0), was incubated at 37°C for 30 min. The mixture was then centrifuged and the supernatant obtained was read at 542 nm to estimate hemoglobin released. Activity was expressed in terms of hemolytic unit (HU), where 1 HU was defined as the amount of peptide inducing 50% hemolysis.

Ichthyotoxicity was examined using killifish *Oryzias latipes* (about 2 cm in total length). Test solutions were prepared by dissolving various amounts of toxin in 50 ml of distilled water. A group of five fish was immersed in each test solution and the mortality was measured after 2 h. The LC_{50} values were calculated by the method of Litchfield and Wilcoxon (1949).

For comparison, the hemolytic and ichthyotoxic activities of melittin (Sigma, St. Louis, USA), the peptide toxin from the bee venom, were similarly determined.

2.4. Analytical methods

Peptide determination was followed by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Thin layer chromatography (TLC) was carried out either on a silica gel plate (type LHP-K; Whatman Japan, Tokyo, Japan) with a solvent system of chloroform–methanol–water (65:25:4, v:v:v) or on a cellulose plate (type LK2; Whatman Japan) with a solvent system of 1-butanol–ethanol–water (4:1:2, v:v:v), essentially as reported by Hashimoto and Oshima (1972). Grammistins were visualized by spraying a 2% horse erythrocyte suspension, ninhydrin reagent or Dragendorff reagent. The molecular weights of the isolated grammistin components were estimated by either MALDI/TOFMS on a Shimadzu/Kratos Kompact MALDI I instrument or gel filtration HPLC on a TSKgel G3000SW column (0.75 × 30 cm; Tosoh, Tokyo) with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). In gel filtration HPLC, ovalbumin, horse myoglobin, ribonuclease A and aprotinin were used as reference proteins. For the determination of amino acid composition, each isolated grammistin was hydrolyzed with 50 µl of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in an evacuated tube at 115°C for 24 h and applied to an Atto MLC-703 amino acid analyzer. Sequence analyses were performed with a gas-phase sequencer (LF-3400D TriCart with high sensitivity chemistry (Beckman, Fullerton, USA)). CD spectra were recorded at 25°C on a JASCO J-720 spectropolarimeter in the range from 190 to 250 nm. In CD experiments, melittin was used as a reference.

3. Results

3.1. Isolation

The crude toxin exhibited hemolytic activities against all the four kinds of

Table 1
Hemolytic and ichthyotoxic activities of grammistins and melittin

Toxin	Hemolytic activity (HU/mg)				LC ₅₀ against killifish (µg/ml)
	horse	cow	sheep	rabbit	
Grammistin					
Crude toxin	130	62	67	73	
Gs 1	131	62	78	84	1.9
Gs 2	1470	700	450	780	0.19
Melittin	145	54	68	76	2.7

animal erythrocytes tested (Table 1). Since horse erythrocytes were about two-fold more sensitive to the crude toxin than the other animal erythrocytes, they were used in hemolytic assays to trace grammistins throughout the following isolation procedure.

When the crude toxin was chromatographed on Sephadex LH-20, hemolytic activity appeared at around fraction 27 as a single peak (Fig. 1A). Active fractions (fractions 25–31) were pooled and then subjected to reverse-phase HPLC on TSKgel ODS-120T, in which a number of peaks were observed as monitored by absorbance at 258 nm (Fig. 1B). Two grammistin components (named grammistins Gs 1 and Gs 2 in the order of elution) were eluted at retention times of 20–24 min (Fr. I) and 26–29 min (Fr. II), respectively, with high concentrations

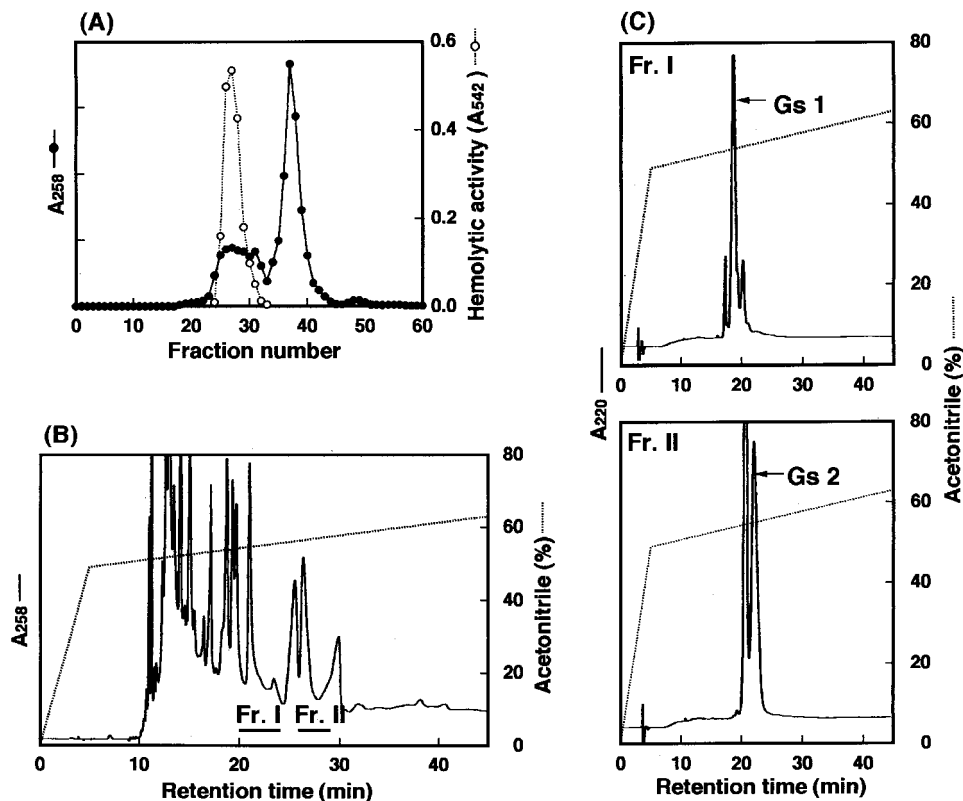


Fig. 1. Isolation of grammistins Gs 1 and Gs 2. (A) Gel filtration on Sephadex LH-20. Sample, crude toxin; column size, 2.5×90 cm; solvent, methanol-1% aqueous acetic acid (3:1, v:v); volume per fraction, 8 ml. (B) Reverse-phase HPLC on TSKgel ODS-120T. Sample, active fraction obtained by gel filtration on Sephadex LH-20; column size, 0.46×25 cm; elution, gradient of acetonitrile in 0.1% TFA; flow rate, 1 ml/min. Two active fractions (Fr. I and II) are indicated by bars. (C) Reverse-phase HPLC on TSKgel ODS-80Ts. Sample, Fr. I (upper) and Fr. II (lower) obtained by reverse-phase HPLC on TSKgel ODS-120T; column size, 0.46×15 cm; elution, gradient of acetonitrile in 0.1% TFA; flow rate, 1 ml/min. Grammistin-containing peaks are indicated by arrows.

of acetonitrile in 0.1% TFA, suggesting their strong hydrophobicity. Since both Frs. I and II were found to be contaminated with impurities by TLC analyses, they were further applied to reverse-phase HPLC on TSKgel ODS-80Ts. As shown in Fig. 1C, impurities were effectively removed and grammistins Gs 1 and Gs 2 were thus isolated. Although only a part of the active fraction obtained by Sephadex LH-20 column chromatography was subjected to reverse-phase HPLC, the yields from 10.6 g of the skin secretion were evaluated to be 70 mg for grammistin Gs 1 and 9.1 mg for grammistin Gs 2 by calculation. Each isolated grammistin gave a single spot on TLC, demonstrating the homogeneity. The R_f values of grammistins Gs 1 and Gs 2 were 0.00 and 0.40, respectively, on a silica gel plate and 0.50 and 0.72, respectively, on a cellulose plate. The two grammistins were positive to both ninhydrin and Dragendorff reagents.

3.2. Hemolytic and ichthyotoxic activities

As shown in Table 1, grammistin Gs 2 showed 6–13× higher hemolytic activity than both grammistin Gs 1 and melittin. Aside from the apparent difference in potency, they displayed similar hemolytic spectra against the different animal erythrocytes; they lysed horse erythrocytes most strongly. In accordance with the hemolytic potencies, grammistin Gs 2 was 10 and 14× higher in ichthyotoxicity against killifish than grammistin Gs 1 and melittin, respectively. At nearly the LC_{50} , the grammistins and melittin killed the fish in about 60 min.

3.3. Structure

Analyses by a sequencer established the complete amino acid sequences of grammistin Gs 1 comprising 25 residues and grammistin Gs 2 comprising 24 residues, as shown in Fig. 2. No impurities were detected in each cycle of the sequencings, supporting the homogeneity of the isolated grammistins. The accuracy of the elucidated sequences was guaranteed by the following two criteria: (1) for both grammistins, the number of each amino acid residue from the sequence coincided well with that from the amino acid analysis, with a slight difference of Pro in grammistin Gs 2 (Table 2); (2) the molecular weights (2790 for

	1									10										20							30						
Grammistin Gs 1	L	F	G	F	L	I	K	L	I	P	S	L	F	G	A	L	S	N	I	G	R	N	R	N	Q								
Grammistin Gs 2	L	F	G	F	L	I	P	L	L	P	H	I	G	A	I	P	Q	V	I	G	A	I	R										
Melittin	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q							
Pardaxin P-1	G	F	F	A	L	I	P	K	I	I	S	S	P	L	F	K	T	L	L	S	A	V	G	S	A	L	S	S	S	G	E	Q	E
Pardaxin P-2	G	F	F	A	L	I	P	K	I	I	S	S	P	I	F	K	T	L	L	S	A	V	G	S	A	L	S	S	S	G	G	Q	E
Pardaxin P-3	G	F	F	A	F	I	P	K	I	I	S	S	P	L	F	K	T	L	L	S	A	V	G	S	A	L	S	S	S	G	E	Q	E

Fig. 2. Amino acid sequences of grammistins Gs 1 and Gs 2 aligned with those of melittin and pardaxins. For grammistins Gs 1 and Gs 2, identical residues are boxed and analogous residues are shaded. Note that the C-terminal residue of melittin is amidated.

Gs 1 and 2570 for Gs 2) calculated from the sequences were very close to those (2792 for Gs 1 and 2572 for Gs 2) determined by MALDI/TOFMS.

In addition to the common features of amino acid compositions (absence of Thr, half-Cys, Met, Tyr and Trp and predominance of hydrophobic amino acids such as Ile and Leu), the sequences of both grammistins were similar to each other. They had the same residues at 10 positions, including the N-terminal 6 successive residues composed mostly of hydrophobic residues and analogous residues at 6 positions (Fig. 2). The significant difference was recognized in the C-terminal region; the segment 20–25 of grammistin Gs 1 was composed of only hydrophilic residues, whereas the corresponding segment of grammistin Gs 2 was rather hydrophobic. It is worth mentioning that the grammistins have no homologies with melittin and pardaxins whose sequences are included in Fig. 2 for convenience.

CD experiments showed that the two grammistins were comparable to melittin as to secondary structure (Fig. 3). All the toxins were judged to be randomly coiled in distilled water. In the presence of SDS micelles, however, they gave typical α -helical spectra with two negative peaks at around 208 and 222 nm. The α -helical contents, calculated by the method of Greenfield and Fasman (1969), were 27% for grammistin Gs 1, 69% for grammistin Gs 2 and 37% for melittin.

On gel filtration HPLC using a TSKgel G3000SW column, both grammistins

Table 2
Amino acid compositions of grammistins Gs 1 and Gs 2

Amino acid	No. of residues ^a	
	grammistin Gs 1	grammistin Gs 2
Asx	2.7 (3)	0.1 (0)
Thr	0.0 (0)	0.0 (0)
Ser	1.8 (2)	0.1 (0)
Glx	1.1 (1)	1.1 (1)
Pro	1.0 (1)	1.9 (3)
Gly	3.3 (3)	3.5 (3)
Ala	1.0 (1)	2.1 (2)
Val	0.0 (0)	0.9 (1)
Cys (half)	0.0 (0)	0.0 (0)
Met	0.0 (0)	0.0 (0)
Ile	2.8 (3)	5.8 (6)
Leu	5.4 (5)	4.4 (4)
Tyr	0.0 (0)	0.0 (0)
Phe	3.0 (3)	2.2 (2)
His	0.0 (0)	0.9 (1)
Trp	0.0 (0)	0.0 (0)
Lys	0.9 (1)	0.1 (0)
Arg	1.9 (2)	1.9 (1)

^a Calculated on the basis that total residues are 25 for grammistin Gs 1 and 24 for grammistin Gs 2. The numbers in parentheses are from sequence data.

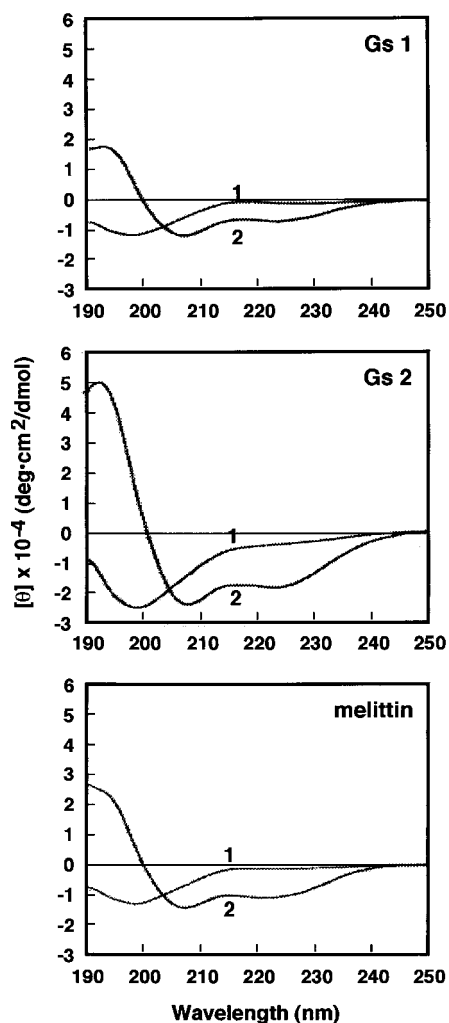


Fig. 3. CD spectra of grammistin Gs 1 (upper), grammistin Gs 2 (middle) and melittin (lower). Solvent: distilled water (1) or 2.5% aqueous SDS (2).

were eluted at the same retention time corresponding to a molecular weight of about 9000. In view of the molecular weights calculated from the sequences, they were likely to exist as aggregates of 3–4 molecules in aqueous solvents, possibly through the interactions of hydrophobic domains.

4. Discussion

In the present study, we isolated two grammistin components (grammistins Gs 1 and Gs 2) with both hemolytic and ichthyotoxic activities from the skin secretion

of *G. sexlineatus* by gel filtration and reverse-phase HPLC and determined their primary and secondary structures. This is the first to report the structures of grammistins having remained to be elucidated for about 20 yr.

Previously, three grammistin components (components I, II and III) have been purified from *G. sexlineatus* by a combination of gel filtration on Sephadex LH-20 and countercurrent distribution (Oshima et al., 1974). Based on the amino acid compositions and the behaviors in Sephadex LH-20 column chromatography and TLC, grammistins Gs 1 and Gs 2 were judged to be identical with components III and II, respectively. In this study, a grammistin corresponding to component I, which was expected to be eluted from a Sephadex LH-20 column later than components II and III, was not detected.

The finding that the *G. sexlineatus* grammistins are simple peptides is of particular importance. All the grammistins previously purified from *G. sexlineatus* (Oshima et al., 1974) and *P. punctata* (Hashimoto and Oshima, 1972; Oshima and Hashimoto, 1976) have been concluded to be peculiar peptides with tertiary amine or quaternary ammonium base moieties, mainly based on the positive reaction to Dragendorff reagent. In this study as well as in the previous studies, grammistins were purified under fairly rigorous conditions using several organic solvents. Consequently, tertiary amine or quaternary ammonium base moieties, if elaborated in grammistin molecules, should be covalently bound to peptide moieties. However, the molecular weights determined by a MALDI/TOFMS technique are very close to those calculated from the amino acid sequences, evidencing that both grammistins Gs 1 and Gs 2 are simple peptides without any other moieties. Our results with the *G. sexlineatus* grammistins strongly suggest that the grammistins from other grammistid fishes and the grammistin-like toxins from coral-gobies (Hashimoto et al., 1974; Shiomi and Hashimoto, 1976) and the clingfish (Hori et al., 1979) are also simple peptides. At present, however, we have no clear explanation for the positive reaction to Dragendorff reagent of the grammistins and grammistin-like toxins.

A search by the National Biomedical Research Foundation database (Release 50.0, September 1996) revealed that the *G. sexlineatus* grammistins do not have sequence homologies with any proteinic or peptidic toxins from other sources. It is, however, interesting to note that the grammistins have many similarities to the following two classes of peptide toxins (see Fig. 2 for their amino acid sequences): melittin composed of 26-residues from the bee venom (Habermann, 1972) and pardaxins of 33-residues from the skin secretion of Pacific sole *Pardachirus pavoninus* (Thompson et al., 1986) and Red Sea Moses sole *P. marmoratus* (Shai et al., 1988; Thompson et al., 1988). All toxins are strongly surfactant, have almost equal chain lengths, display hemolytic and ichthyotoxic activities, exist mainly as α -helices in the presence of SDS micelles and aggregate into multimeric forms comprising 3–4 molecules.

In relation to the surfactancy, the major secondary structure in the presence of SDS micelles has been shown to be amphiphilic α -helix in common with melittin (Dawson et al., 1978) and pardaxins (Zagorski et al., 1991). This appears to be the case with the grammistins. Since it is now unclear which segments of the

grammistins are α -helical, their amphiphilicity was evaluated by plotting the entire amino acid sequences on helical wheels (projections of the amino acid side-chains onto a plane perpendicular to the axis of the helix) according to the method of Schiffer and Edmundson (1967). As shown in Fig. 4, grammistins Gs 1 is capable of forming an amphiphilic structure in the segment from positions 1–19; one face of the segment is occupied by only hydrophobic residues, while all hydrophilic residues are located at the opposite side chain. The α -helical portions are presumably contained in this amphiphilic segment. In contrast, grammistin Gs 2 can be amphiphilic throughout the entire chain, implying that the α -helical portions of grammistin Gs 2 are completely amphiphilic. It should be pointed out that the α -helical content (69%) of grammistin Gs 2 in micelles is much higher than that (37%) of melittin as well as that (27%) of grammistin Gs 1. Moreover, the α -helical content of pardaxins was previously estimated to be 39% under the same condition as adopted in this study (Thompson et al., 1986). Thus, grammistin Gs 2 is unique in that it is significantly abundant in amphiphilic α -helicity.

The similarity between the grammistins and the other two classes of peptide toxins was further evidenced by the Eisenberg plot (Eisenberg et al., 1984). As shown in Fig. 5, the grammistins, like both melittin and pardaxins, locate in the surface seeking region, suggesting that they have a tendency to bind lipid membranes lying flat at their surface. It is, therefore, very likely that the grammistins elicit their biological activities by the same mechanism as known for melittin (Habermann, 1972) and pardaxins (Tachibana et al., 1989; Rapaport and Shai, 1991), that is, 3–4 molecules of the grammistins assemble in the lipid bilayer and form pores. Indeed, our preliminary experiments have revealed that the hemolytic activity of the grammistins is inhibitable by several phospholipids such as phosphatidylcholine and sphingomyelin. The variable susceptibilities of the four kinds of animal erythrocytes against the grammistins (Table 1) seem to be explained by the different lipid compositions of the erythrocyte membranes.

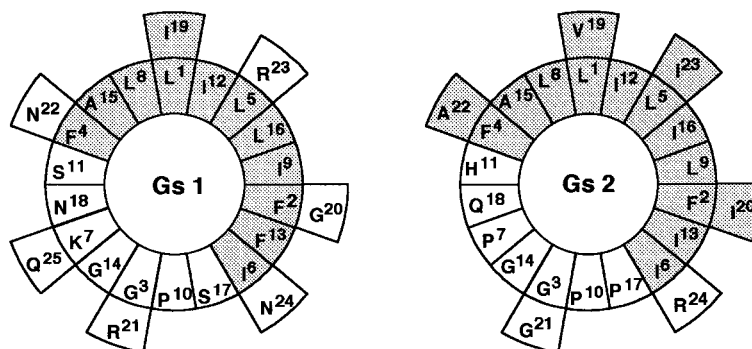


Fig. 4. Helical wheel projections of grammistin Gs 1 (left) and grammistin Gs 2 (right). Shaded areas represent hydrophobic residues and the rest hydrophilic residues.

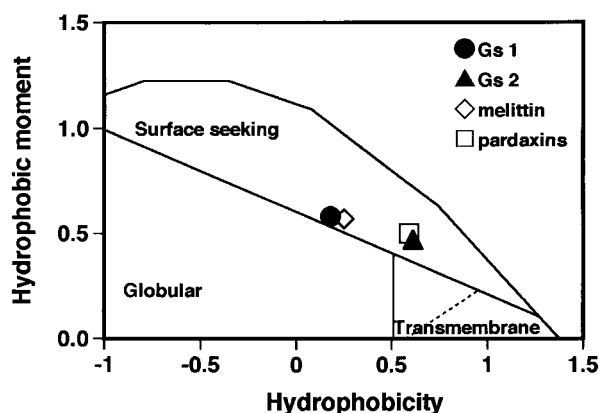


Fig. 5. Eisenberg plot of grammistins Gs 1 and Gs 2, melittin and pardaxins. Mean hydrophobicity and mean hydrophobic moment were calculated using a window of 11 residues by the method of Eisenberg et al. (1984).

Finally, it should be emphasized that the grammistins exhibit higher biological activities than melittin and pardaxins. This study demonstrated that grammistin Gs 2 is about 10× higher in both hemolytic and ichthyotoxic activities than melittin, although grammistin Gs 1 shows the same magnitude in potencies as melittin. As compared to the minimum lethal dose (10 µg/ml) against killifish (the same species used in this study) reported for both pardaxins P-1 and P-2 (Thompson et al., 1986), grammistin Gs 1 (LC_{50} 1.9 µg/ml) and Gs 2 (LC_{50} 0.19 µg/ml) are 5 and 50× higher in ichthyotoxicity, respectively. The markedly high biological activities of grammistin Gs 2 appear to be associated with its peculiar secondary structure, i.e. extraordinary abundance in amphiphilic α -helicity. Thus, the grammistins, especially grammistin Gs 2, will be a new valuable tool in investigating the mode of action of surface seeking peptides. In this relation, it is important to synthesize them and clarify their detailed structures by more reliable techniques such as NMR.

Acknowledgements

The authors thank Dr. A. Shinagawa, Gakushuin Women's College and Dr. S. Ishizaki, Tokyo University of Fisheries, for measuring the amino acid compositions and CD spectra of the isolated grammistins, respectively.

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